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2019-05

Knuuttila , M , Hämäläinen , E & Poutanen , M 2019 , ' Applying mass spectrometric methods to study androgen biosynthesis and metabolism in prostate cancer ' , Journal of Molecular Endocrinology , vol. 62 , no. 4 , pp. R255-R267 . <https://doi.org/10.1530/JME-18-0150>

<http://hdl.handle.net/10138/301526>

<https://doi.org/10.1530/JME-18-0150>

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REVIEW

Applying mass spectrometric methods to study androgen biosynthesis and metabolism in prostate cancer

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Abstract

Recent development of gas chromatography and liquid chromatography-tandem mass spectrometry (GC-MS/MS, LC-MS/MS) has provided novel tools to define sex steroid concentrations. These new methods overcome several of the problems associated with immunoassays for sex steroids. With the novel MS-based applications we are now able to measure small concentrations of the steroid hormones reliably and with high accuracy in both body fluids and tissue homogenates. The sensitivity of the tandem mass spectrometry assays allows us also for the first time to reliably measure picomolar or even femtomolar concentrations of estrogens and androgens. Furthermore, due to a high sensitivity and specificity of MS technology, we are also able to measure low concentrations of steroid hormones of interest in the presence of pharmacological concentration of other steroids and structurally closely related compounds. Both of these features are essential for multiple preclinical models for prostate cancer. The MS assays are also valuable for the simultaneous measurement of multiple steroids and their metabolites in small sample volumes in serum and tissue biopsies of prostate cancer patients before and after drug interventions. As a result, novel information about steroid hormone synthesis and metabolic pathways in prostate cancer has been obtained. In our recent studies, we have extensively applied a GC-MS/MS method to study androgen biosynthesis and metabolism in VCaP prostate cancer xenografts in mice. In the present review, we shortly summarize some of the benefits of the GC-MS/MS and novel LC-MS/MS assays, and provide examples of their use in defining novel mechanisms of androgen action in prostate cancer.

Key Words

- ▶ prostate
- ▶ mass spectrometry
- ▶ testosterone
- ▶ dihydrotestosterone
- ▶ immunoassay

*Journal of Molecular
Endocrinology*
(2019) **62**, R255–R267

Development of highly sensitive mass spectrometric assays for analyzing androgens in body fluids and tissues

To provide solid evidence for the role of local steroid metabolism as a key regulatory step in sex steroid biology we and several other research groups have actively developed liquid chromatography-tandem mass spectrometric (LC-MS/MS) and gas chromatography (GC)-MS/MS methods to analyze multiple unconjugated and/or conjugated steroid hormones simultaneously in body fluids and tissue biopsies. Indeed, the value of MS assays in obtaining novel data in both clinical specimens and preclinical models is well demonstrated (e.g. [Vandenput *et al.* 2007](#), [Harwood & Handelsman 2009](#), [Arlt *et al.* 2011](#), [Keski-Rahkonen *et al.* 2011](#), [Keski-Rahkonen *et al.* 2013](#), [Tamae *et al.* 2013](#), [Ke *et al.* 2015](#), [Nilsson *et al.* 2015](#), [Boggs *et al.* 2016](#), [Snaterse *et al.* 2016](#), [Zang *et al.* 2017](#), [Häkkinen *et al.* 2018](#)). The discussion and debate for the benefits and drawbacks of steroid hormone analysis using either MS or immunoassay methodology has been active, and well-summarized in recent reviews ([Taylor *et al.* 2015](#), [Cross & Hornshaw 2016](#), [Handelsman 2017](#), [Wudy *et al.* 2018](#)). However, while validated immunoassays may be adequate for the screening of serum testosterone (T) concentrations in the normal physiological range of men ([Taylor *et al.* 2015](#)), problems with sensitivity, specificity and reproducibility of the direct immunoassays emerge for most androgens and estrogens when those are applied to low levels, such as in postmenopausal women, children and men with suppressed gonadal function. While the performance of the immunoassays may be improved by using liquid or solid-phase extraction and chromatographic separation, or other preanalytical sample handling procedures before the immunoassay, the use of these procedures are often abolished, because the benefits of automatization and high throughput of these assays are then lost. Furthermore, it is very difficult and laborious to produce specific, high-affinity antibodies against all steroids and their metabolites, but almost all of the steroids can be ionized, and they give reasonable fragmentation in MS, so they can be analyzed and quantitated with MS/MS assays.

Sensitivity of MS/MS assays are superior to immunoassays (picomolar to femtomolar levels of steroids vs nanomolar to picomolar levels with immunoassays). Most commercial direct immunoassay of steroids (luminometric or immunofluorometric methods) do not measure steroid levels reproducibly below 20 pmol/L, and moreover, the accuracy of the immunoassays compared to reference methods is low at these low levels. For example,

the reproducibility of direct estradiol assays below the concentration of 100 pmol/L is often unacceptable (more than 20% CV). Furthermore, reproducibility of LC-MS/MS is better than in most steroid immunoassays in a large concentration range. Moreover, stable isotope-labeled internal standards with physicochemical properties similar to the analytes are used to increase the precision and accuracy of the MS/MS methods, leading to improved intra-injection reproducibility and reduction of matrix and ionization effects ([Bergeron *et al.* 2009](#), [Bronsema *et al.* 2012](#)).

For clinical purposes, MS assays and immunoassays are currently at very different phases of their maturity. While immunoassays have been performed for decades in routine laboratories, the technical and methodological development of MS/MS methods has been rapid during the recent 10 years. The high initial equipment cost and the costs of maintenance are often considered to be significant obstacles to MS technique use in research laboratories. Compared to immunoassay, MS methodology also needs special technical expertise, which can be difficult to obtain, if the laboratory has no previous experience of chromatographic techniques or MS technology. However, the development of user-friendly software and easy accessible structures of MS equipment have made the operation and their routine maintenance rather easy. Thus, in many laboratories LC-MS/MS and GC-MS/MS equipment are run routinely alone by educated laboratory technicians after the validation of the method by chemists. Moreover, tested and pre-validated methods for steroid analyses are nowadays available for certain LC-MS/MS instruments by the instrument manufacturers. These are available for both single analytes (testosterone (TS), estrone, estradiol) and steroid profiles.

The high-throughput and extensively automatized procedures reducing the costs have been the benefits of the immunoassays applied in clinical laboratories and big study cohorts. However, the development of preanalytical pipetting automation for solvent transfer and liquid-liquid or solid-phase extraction, and the use of multiplexing techniques with synchronized injections of two to four HPLC systems incorporated to single MS unit, has become an effective tool for improving throughput of MS methods ([Bayliss *et al.* 2000](#), [Jannetto & Fitzgerald 2016](#)). The multiplexing technique utilizes the fact that in most of the MS assays the analyte or analytes of interest are typically separated by a few seconds so that the chromatographic run is completed within some minutes. By combining profiling method and multiplexing technique makes it possible to further increase the capacity

of the MS method, and significantly reduce the cost of a single sample to a level which is competitive compared with any steroid immunoassay. Thus, it seems that the use of immunoassays for steroid hormone analysis is rapidly reducing, and in the future will be limited mainly to some automated routine methods and screening purposes in clinical laboratories.

It is evident that MS assays have significantly improved the specificity of steroid measurements. This is largely due to the fact that MS methodology gives direct structural data of the analyte which cannot be obtained with immunological techniques. Thus, for the steroid research, the MS/MS assays are the methods of choice already today for measuring the serum and tissue concentrations of sex steroids in many preclinical models. Measuring steroids in mice and rats is even more challenging than in humans, due to their lower concentrations in rodents (Nilsson *et al.* 2015). One of the reasons for the low circulating sex steroid levels in rodents is that, in contrast to human, they do not present with sex steroid hormone-binding globulin (SHBG) in the circulation. In primates, SHBG binds to circulating sex steroids and limits the amount of free form of the steroids available for target tissues. It was recently demonstrated that expression of SHBG in transgenic mice, indeed, increases the total serum level of TS (Laurent *et al.* 2016), providing a convincing proof for the free hormone theory as one of the feedback mechanism for the regulation of sex steroid concentrations in circulation. While validated immunoassays for TS measurement (Huhtaniemi *et al.* 1985) in male mice have provided quite an acceptable performance, none of the direct immunoassays has been sensitive or reliable enough, for example to measure normal physiological (picomolar) levels of estradiol in mice. Thus, one should be cautious when conclusions are drawn from studies where estradiol concentration in mice has been measured with immunoassays, and we advise that any critical study should be confirmed by high-sensitivity MS/MS assays.

As addressed above, one significant problem associated with the steroid immunoassays is the cross-reactivity of the antibodies with other closely related steroids. Although the cross-reactivity is not a significant obstacle in many clinical studies with high physiological concentrations of the steroid hormones, even a low cross-reactivity becomes a problem in any clinical or preclinical study that involves administration of a pharmacological or supraphysiological dose (Husen *et al.* 2006, Savolainen *et al.* 2007) of a steroidal compound. Unlike immunoassays, MS techniques can be used to measure

the steroids with high specificity also with big differences in the concentrations of the related steroids. In addition, the dynamic range of the physicochemical MS methods is superior compared to immunoassays.

Poor ionization properties of certain steroids have been one of the challenges in some MS methods, leading to low sensitivity of these assays. Thus, derivatization of the steroids has often been a prerequisite procedure for improving the sensitivity (Keski-Rahkonen *et al.* 2011, Häkkinen *et al.* 2018), but it also increases the complexity, reproducibility and the workload of the method. Nevertheless, the current LC-MS/MS-based assays without derivatization provide very high sensitivity with reference level specificity, also with multianalyte methods; however, neither with MS methodology is it simple to guarantee the right or accurate result of certain steroid. Especially one must be careful when analyzing steroids in lipid-rich tissues using MS methods (Liere & Schumacher 2015). All the phases of the analysis must be carefully tested, including efficiency of the extraction of the steroids (liquid-liquid or solid-phase), solubility of steroids to organic or water-based solvents and buffers used and the efficiency in the purification and separation with chromatographic procedures. Especially, the deconjugation of steroid conjugates via solvolysis or hydrolysis during the process and the conversion of steroids to their metabolites must be carefully tested for proper quantitation of steroids using MS assays. Taking the issues above into a count, with the state-of-the-art GC- and LC-MS/MS techniques, we can now measure several androgens with methods being sensitive enough to most applications both in human and preclinical studies (see, for example Harwood & Handelsman 2009, Nilsson *et al.* 2015, Häkkinen *et al.* 2018). However, different assay conditions are often needed for the maximal sensitivity for different steroids, and the techniques used need to be selected based on the preferred sensitivity for the steroids of highest interest (Table 1, for examples).

Due to the well-acknowledged benefits in using MS/MS-based sex steroid assays in scientific research, the editorial board of the *Journal of Clinical Endocrinology and Metabolism* stated that any study published in the journal from year 2015 onwards should use MS methods for the measurement of sex steroids (Handelsman & Wartofsky 2013). This further increased the debate on the pros and cons of such a decision amongst endocrinologists. In response to this debate, The Endocrine Society Council then brought together a Sex Steroid Assays Reporting Task Force in order to recommend the future reporting of sex steroid hormone measurements in the Society's journals.

Table 1 Mass spectrometric assays recently applied by us to measure serum and intratissue androgen levels in preclinical models and clinical specimens.

	LC-MS/MS ^a	GC-MS/MS ^b	LC-MS/MS ^c
Estrone	1.5	0.5	0.5
Estradiol	2.5	0.5	2.0
Testosterone	10	8	1
DHT	50	2.5	–
3 α Diol	200	–	–
3 β Diol	200	–	–
Androstenedione	–	12	4
Progesterone	–	74	4
DHEA	–	400	100
17 α -hydroxy-progesterone	–	–	10
17 α -hydroxy-pregnenolone	–	–	10

^aHarwood and Handelsman (2009); ^bNilsson *et al.* (2015); ^cHäkkinen *et al.* (2018).

The instructions provided did not mandate a specific type of assay for steroids, likely due to the unavailability of new GC- or LC-MS/MS methods to a large group of scientists in the field. However, the recommendation stated the criteria for a valid assay for sex steroids in clinical and nonclinical studies. For example, the commercial sex steroid assays validated for human serum must be validated to verify acceptable quantitative accuracy, precision and specificity for the analytes measured when applied in nonclinical studies performed *in vitro* or in animals. It was also recognized that automated and direct (unextracted) immunoassays for sex steroids might present with unacceptable bias when concentrations are low, for example in samples from children, women, hypogonadal men and postmenopausal women (Wierman *et al.* 2014).

We expect that the new multianalyte profiling of steroids by GC-MS/MS and LC-MS/MS methods will result in a new era in steroid biology. They will bring tremendous possibilities to discover novel ligands and functions for classical sex steroids, and enable us to identify biomarker combinations for diagnostic purposes, patient stratification and the treatments of endocrine diseases.

Detecting novel androgenic ligands with mass spectrometric assays

Although TS and dihydrotestosterone (DHT) are the primary androgens activating the androgen receptor (AR), many of the precursors for these classical androgens and their metabolites are also capable of AR binding. MS/MS methods have been critical in identifying these novel steroids, and methods are now available to measure their concentrations in serum, providing also clinical value.

Furthermore, AR mutations may also reduce the ligand specificity, causing promiscuous activation of the AR with antiandrogens, estrogens, progestins and glucocorticoids (Coutinho *et al.* 2016).

Besides the classical adrenal androgens, androstenedione (A-dione), dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S), MS assays have shown that human adrenals produce 11 β -hydroxyandrostenedione (11OHA-dione) from A-dione by CYP11B hydroxylase activity (Bloem *et al.* 2013, Swart *et al.* 2013). Although 11OHA-dione has no significant androgenic activity (Bélanger *et al.* 1993), it can be metabolized to 11-ketoandrogens, namely to 11-ketotestosterone (11KT) and 11-ketodihydrotestosterone (11KDHT) that present with androgenic properties (Storbeck *et al.* 2013, Pretorius *et al.* 2016). These 11-ketoandrogens can be formed when the necessary enzymatic machinery is present, including 5 α -reductase, 17 β -HSD and 11 β -HSD activities (Swart & Storbeck 2015). Both 11KT and 11KDHT bind to the AR with potencies similar to T and DHT, respectively, and induce changes in androgen-regulated gene expression and enhance androgen-dependent cell proliferation (Pretorius *et al.* 2016). In addition to the findings indicating that 11-ketoandrogens are derived from 11OHA-dione of adrenal origin, a recent study suggested that 11KT is extensively produced by the Leydig cells of the testes and also by the ovarian theca cells (Imamichi *et al.* 2016), thus, being a gonad-produced sex steroid. However, the plasma concentrations of 11OHA-dione, 11KA-dione, 11OHT and 11KT measured by LC-MS/MS are significantly increased in 21-hydroxylase (CYP21A)-deficient patients, supporting the hypothesis that these steroids are originating from adrenal precursors (Turcu *et al.* 2016). Interestingly, remarkable levels of 11KT and 11KDHT were also detected in prostate tissue and plasma of prostate cancer (PCa) patients by LC-MS/MS (du Toit *et al.* 2017), suggesting their potential importance in androgen-dependent tumor growth particularly in castration-resistant prostate cancer (CRPC).

The 11-ketoandrogens are not the only novel ligands that can activate the wild-type AR. For example, 11-deoxycorticosterone (DOC), a precursor of corticosterone, has been identified as a novel substrate for Steroid 5 Alpha-Reductase 1 (SRD5A1), and DOC and its 5 α -reduced metabolite 5 α -dihydrodeoxycorticosterone (5 α -DH-DOC) was demonstrated to promote cell proliferation through AR in PCa cell lines (Uemura *et al.* 2010). Furthermore, LC-MS/MS measurements have confirmed that 5 α -DH-DOC is present in tumors obtained from CRPC patients.

Novel enzymes for androgen biosynthesis

Data from recent years have provided convincing evidence indicating that the ligand concentration available for steroid receptor binding is markedly regulated by the target tissue metabolism. The 5 α -reductase activity is a classic example of this phenomenon, as conversion of T to DHT has been known for a long time to be essential for prostate development and prostate hyperplasia (Mendonca *et al.* 2017, Banerjee *et al.* 2018, Matsushita *et al.* 2018). 5 α -reductase inhibitors, finasteride and dutasteride, are commonly described drugs for the treatment of benign prostatic hyperplasia. Finasteride specifically inhibits 5 α -reductase type 1 (SRD5A1) whereas dutasteride effectively inhibits both SRD5A1 and SRD5A2 enzymes (Clark *et al.* 2004, Kim *et al.* 2018). However, 5 α -reductase inhibitors have been shown to lead to accumulation of intratumoral T (Byrns *et al.* 2012, Knuuttila *et al.* 2018b) that has reduced the use of these compounds in PCA therapy. However, 5 α -reductase inhibitors may also slow down the progression of PCa (Cha & Shariat 2011), while their role in PCa prevention remains controversial. Based on two big randomized trials, 5 α -reductase inhibitors decrease the incidence of PCa but also increase the risk of high-grade tumors, yet the relevance of these associations are under debate (Lacy & Kyprianou 2014).

Equally, we know that in the postmenopausal women, majority of the estrogens are produced in peripheral tissues from the androgenic precursors produced in the ovaries and adrenals (Labrie 1991). The extragonadal estradiol production is highly related to the expression of P450 aromatase (Cytochrome P450, family 19) in peripheral tissues (Simpson 2002). In line with these findings, P450 aromatase inhibitors have become key players in the endocrine therapy of estrogen-dependent breast cancer in postmenopausal women (Santen *et al.* 2009, Zahid *et al.* 2016). However, current data suggest that these are just a few examples of multiple similar physiological settings in which the local sex steroid synthesis and metabolism is in a key position to regulate the hormone action both in men as well as in premenopausal and postmenopausal women.

The annotation of the whole human genome has provided evidence for the presence of up to 70 enzymes in the short-chain dehydrogenase/reductase (SDR) family (Persson *et al.* 2008), up to 15 enzymes in aldoketo reductase (AKR) family (Penning 2015) and about 50 CYP family enzymes (Nebert *et al.* 2013). In all these families, there are enzymes demonstrated to be involved in the metabolism of hydrophobic small molecules of

various origins, and steroid-metabolizing enzymes have been identified in all these enzyme families. Although the catalytic properties of SDR (Oppermann *et al.* 1997, Wu *et al.* 2007) and AKR (Jin & Penning 2007, Penning & Drury 2007) enzyme families have been studied, some of the enzymes in these families are still poorly characterized, providing a large pool of potential novel steroid-metabolizing enzymes. This hypothesis is supported by the fact that new enzymes with steroid-metabolizing activities and novel reactions for known steroid-metabolizing enzymes are constantly identified (Neunzig *et al.* 2015, van Rooyen *et al.* 2018). These novel steroid-synthesizing enzymes and new activities associated with the classical enzymes have led to the identification of nonclassical pathways for the production of active sex steroids, including androgens (Miller 2017, Mohler 2018, Schiffer *et al.* 2018).

Androgen biosynthesis inhibitors serve as an effective approach to treat prostate cancer

The identification of novel tissues and pathways involved in sex steroid synthesis is also the basis for the development of new therapies affecting sex steroid-dependent diseases. Abiraterone acetate is a steroid synthesis inhibitor that is currently used in clinical practice to treat patients with CRPC. Abiraterone acetate is metabolized *in vivo* to abiraterone that inhibits the 17 α -hydroxylase and 17,20-lyase activity of CYP17A1, resulting to a marked, up to 90%, decrease of serum T levels in both castrated and non-castrated patients (O'Donnell *et al.* 2004, Attard *et al.* 2005, Ryan *et al.* 2014). Treatment with abiraterone acetate has been shown to extend the overall survival of metastatic CRPC patients who had previously received chemotherapy for 3–4 months (de Bono *et al.* 2011). Treating patients with metastatic CRPC with abiraterone acetate without previous chemotherapy was also shown to be effective by improving the radiographic progression-free and overall survival (Ryan *et al.* 2013). Interestingly, while abiraterone acetate treatment markedly reduces adrenal androgen concentrations in the serum of patients, DHEA-S still persists in circulation, possibly serving as a precursor for intratumoral conversion to T and DHT in prostate tumors (Taplin *et al.* 2014, Tamae *et al.* 2015). Strikingly, a novel abiraterone metabolite (Δ 4-abiraterone) was recently identified to inhibit HSD3B1 and SRD5A enzymes, and was also shown to act as an AR antagonist, suggesting several independent mechanisms of abiraterone action with potential clinical importance (Li *et al.* 2015).

Furthermore, there are other potential ways to inhibit intratumoral androgen biosynthesis. For example, inhibitors for AKR1C3 have been developed to reduce the conversion of A-dione to T in CRPC tumors (Byrns *et al.* 2011). However, the development of selective AKR1C3 inhibitors is challenging as the AKR1C3 shares up to 86% identity to its homologs, AKR1C2 and AKR1C1, that inactivate DHT in the prostate (Penning *et al.* 2000). Nevertheless, some compounds, including indomethacin (Bauman *et al.* 2005), ASP952 (Kikuchi *et al.* 2014) and GTx-560 (Yepuru *et al.* 2013) inhibit AKR1C3 activity with strong selectivity over AKR1C1 and AKR1C2. The compounds have provided promising results in preclinical studies *in vivo*, thus, being possible future CRPC therapies. Recently, also CYP11A1 inhibitors have been developed toward reducing local androgen biosynthesis in patients with CRPC (Oksala *et al.* 2017).

VCaP xenografts as a preclinical model for prostate cancer

Androgen biosynthesis in PCa is a complex process, regulated by multiple organ interactions. Thus, cell-based models or even primary tissue cultures *in vitro* are not able to well-mimic the physiology typically involved in intratumoral androgen biosynthesis. The benefit of the *in vivo* models is evident, due to the presence of the blood circulation delivering the active androgens and their precursors potentially synthesized by other organs, and due to the presence of the regulatory systems involving multiple endocrine organs and their interactions, including but not limited to the pituitary, adrenals and testes. Thus, still to date, xenograft studies carried out by grafting established PCa cell lines in immunodeficient mice is a highly appropriate method to model intratumoral androgen biosynthesis and metabolism in PCa. These studies aid drug development and help us to understand the growth mechanisms of primary PCa, as well as to model the hormone dependency of CRPC growth. At best, these studies, complemented with studies on androgen profiles in clinical samples obtained from PCa patients, are highly valuable in order to identify mechanisms of androgen biosynthesis during the different disease stages and drug interventions.

One of the PCa models utilized by us and others, both *in vitro* and *in vivo*, is based on VCaP PCa cells. The cell line was originally derived from a vertebral metastatic lesion of a patient with hormone refractory PCa (Korenchuk *et al.* 2001). The VCaP cells are androgen-dependent and express several characteristics typical to human PCa. For

example, the VCaP cells secrete PSA, and express wild-type AR as well as several of the AR splice variants typically present in clinical PCa. Furthermore, similar to advanced PCa, the AR gene is amplified in the VCaP cells, resulting to a high expression of both AR mRNA and protein (van Bokhoven *et al.* 2003, Knuuttila *et al.* 2014). The VCaP cells are suitable for efficient tumor establishment *in vivo* due to high take rate when engrafted either subcutaneously or orthotopically in the prostate (Korenchuk *et al.* 2001, Knuuttila *et al.* 2014). Even more importantly, the VCaP cells grow in intact mice in androgen-sensitive manner, while castration induces a transient reduction in growth rate and a dramatic drop in the PSA secretion (Fig. 1). However, within a few weeks after castration, the tumor growth recurs, mimicking the castration-resistant growth of clinical tumors (Loberg *et al.* 2006, Knuuttila *et al.* 2014, Moilanen *et al.* 2015, Oksala *et al.* 2018).

In contrast to most of the PCa cell lines, including the often used models based on PC-3, LNCaP and DU145 cells, the VCaP cells contain the *TMPRSS2-ERG* fusion gene that is a hallmark of up to 50% of the PCas. Even though, the final role of the oncogenic *TMPRSS2-ERG* fusion gene in PCa remains to be determined, it has been shown to result in overexpression of the ERG oncoprotein that, in turn, has been proposed to lead into epithelial-to-mesenchymal transition and to increased migration and invasion (Archer *et al.* 2017). Furthermore, compared to LNCaP and C4-2 cells, VCaP cells express higher levels of steroidogenic enzymes that are associated with the androgen biosynthesis of clinical CRPC, including AKR1C3, CYP11A1, HSD17B3, HSD17B6, HSD3B2 and SRD5A1 (Cai *et al.* 2011). The above described features of the VCaP cell line make it an appropriate model for studying intratumoral androgen biosynthesis in the different stages of PCa (Fig. 1).

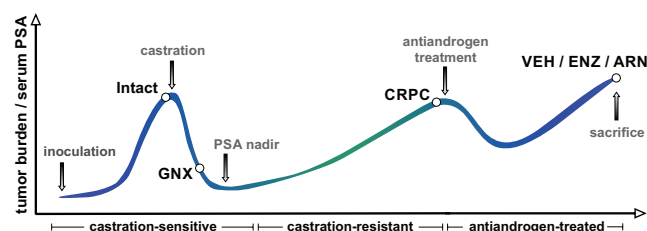


Figure 1

A timeline of the VCaP xenograft growth in nude mice followed by serum PSA, resembling the stages of clinical CRPC. The tumors are inoculated in intact mice, and after 4–5 weeks the tumors are established. The castration causes a dramatic drop in serum PSA, but recurrence of the tumors is observed within 5–6 weeks. After the establishment of the castration-resistant tumors, a response to antiandrogens (ARN, ARN-509; ENZ, enzalutamide) is observed; however, the response seems to be transient.

expression profiling showed that these enzymes were not substantially expressed in the castration-resistant VCaP tumors (Knuuttila *et al.* 2014). The lack of these enzymes essential for *de novo* androgen biosynthesis, together with the MS data indicating measurable levels of A-dione and progesterone in serum of castrated mice (Knuuttila *et al.* 2014), further suggest that the androgens present in the castration-resistant VCaP tumors are, at least partially, synthesized from the precursors of adrenal origin. In line with this, our recent data have shown a reduced growth rate and an antiandrogenic effect in target gene expression in castration-resistant VCaP tumors after adrenalectomy (Huhtaniemi *et al.* 2018).

Particularly, the DHT concentrations were high in the castration-resistant VCaP tumors, whereas the intratumoral T concentrations were clearly lower (Knuuttila *et al.* 2014). We, thus, hypothesize that DHT synthesis in these tumors applies a pathway, being independent of T as an intermediate. Also in other MS-based studies T-independent conversion of A-dione to DHT via androstane-17-one has been demonstrated to be the predominant route for DHT formation in CRPC (Chang *et al.* 2011, Dai *et al.* 2017), while both T-dependent and independent DHT formation are equally used in the primary tumors (Dai *et al.* 2017). Similarly to that observed in VCaP xenografts, intratumoral DHT concentration was retained or increased, after castration in 80% (14/18) of the LuCaP xenografts tested, while T was decreased, suggesting intratumoral DHT synthesis also in majority of these castration-resistant patient-derived xenografts (Nguyen *et al.* 2017). Furthermore, it has been shown that the conversion from A-dione to androstane-17-one is preferably mediated by SRD5A1 and not by SRD5A2 that is the major 5 α -reductase present in the benign prostate (Luu-The *et al.* 2008, Chang *et al.* 2011). Interestingly, *SRD5A1* is also expressed in the castration-resistant VCaP tumors, while no *SRD5A2* expression was detected in these tumors. It is also worth noting that A-dione and T do not present with substrate competition in a 5 α -reductase activity assay applied on cultured prostatic tissue *ex vivo* (Dai *et al.* 2017).

Our recent studies have indicated that the expression of steroidogenic enzymes AKR1C3 and HSD17B6 are upregulated in the castration-resistant VCaP tumors. The activity of AKR1C3 has been frequently associated with the progression of CRPC, thus, it has become a potential novel target for developing inhibitors for intratumoral androgen biosynthesis (Stanbrough *et al.* 2006, Pfeiffer *et al.* 2011, Powell *et al.* 2015). Similar to HSD17B3, AKR1C3 catalyzes the conversion of A-dione and androstane-17-one to T and

DHT, respectively. The expression of *AKR1C3* is abundant in the castration-resistant VCaP tumors, while *HSD17B3* is absent in VCaP cells. Thus, of these two reductive HSD17B enzymes, AKR1C3 is more likely involved in the DHT synthesis in castration-resistant VCaP tumors, either via classical or other pathways (Fig. 2). Alike, HSD17B6 has been suggested to have an important role in the DHT synthesis in CRPC by converting androstane-17-one to DHT (Bauman *et al.* 2006, Mohler *et al.* 2011a,b), however, its enzymatic properties are only poorly characterized. In conclusion, the MS data on steroids suggest that a testosterone-independent pathway is the preferred pathway for DHT synthesis in the castration-resistant VCaP tumors. Further studies with a broader profile of steroids, including the backdoor pathway metabolites, should be conducted using MS-based assays to fully confirm the required androgen biosynthesis machinery in this preclinical CRPC model.

Intratumoral androgen levels are reduced in antiandrogen-treated VCaP tumors

The use of the first potent antiandrogens developed several decades ago has been limited due to severe side effects and interactions with other steroid receptors. However, enzalutamide, a second-generation antiandrogen, has been approved by FDA for the treatment of CRPC. Enzalutamide possesses numerous improved properties compared to the first-generation compounds: it binds to the AR with a greater affinity, blocks the AR more effectively, impairs the ability of the receptor to bind DNA and reduces the nuclear translocation of AR (Tran *et al.* 2009). A similar strong antiandrogenic efficacy is associated with ARN-509 (apalutamide), a derivative of enzalutamide (Clegg *et al.* 2012). Apalutamide has demonstrated antitumor activity in nonmetastatic CRPC patients (Smith *et al.* 2016). In addition, another novel antiandrogen has been recently developed (ODM-201, darolutamide) that has passed successfully phase 1 and 2 trials (Fizazi *et al.* 2014). Importantly, ODM-201 has been demonstrated to block also the action of several AR mutants, such as F876L, thus, having additional features over those present in enzalutamide and ARN-509 (Moilanen *et al.* 2015, Borgmann *et al.* 2018). Partly due to the discovery of AR splice variants that lack the ligand-binding domain (LBD), the need for antiandrogens that target other parts of AR than LBD has also emerged.

To further improve the treatment outcome of patients with CRPC, we have to understand in detail the mechanisms of action of these new antiandrogens.

Interestingly, our recent data obtained by the GC-MS/MS (Fig. 3) demonstrated that antiandrogen treatment for 4 weeks markedly reduced the levels of intratumoral T and DHT in the castration-resistant VCaP xenografts (Knuuttila *et al.* 2018a). This interesting observation could be a result of different mechanisms, one possibility being a direct or indirect effect of the antiandrogens on the intratumoral androgen biosynthesis. This is supported by the observation that treatments with enzalutamide and ARN-509 altered the expression of several steroidogenic enzymes, including *AKR1C2*, *AKR1C3* and *HSD17B6*. Of these, *AKR1C3* is best characterized, and it has been associated with DHT synthesis in CRPC in several studies, as described above. Furthermore, *AKR1C2* has been shown to metabolize DHT to a weak androgenic 3α -diol (Bauman *et al.* 2006), and thus, the reduced DHT concentrations measured could be a result of enhanced conversion of DHT to androstenediol (Fig. 2), a metabolite that could not be detected with high enough sensitivity with our currently available LC-MS/MS or GC-MS/MS methods. Moreover, *HSD17B6* has been often associated with DHT metabolism (Ishizaki *et al.* 2013), while its enzymatic properties are still to be better characterized (Zhang *et al.* 2016). There is also a possibility that these antiandrogens inhibit steroidogenic enzymes directly by competing at the active site with the substrates. This hypothesis

is supported by the findings showing that a metabolite of abiraterone possesses antiandrogenic properties by binding directly to AR (Li *et al.* 2015).

While the VCaP model displays several hallmarks of CRPC, presently it is not known whether the effects of antiandrogens on intratumoral androgen levels translate into clinical CRPC. However, a recent study demonstrated that patients treated for 3 months with GnRH agonist or antagonist combined with antiandrogen (bicalutamide) prior to radical prostatectomy were associated with lower intratumoral DHT levels measured by LC-MS/MS compared to the patients who were treated with one of the GnRH antagonists only (Sayyid *et al.* 2017). No differences in the intratumoral T levels were observed between the treatment groups in this study. However, the lack of sensitivity in the MS assay used might have affected the results. In summary, the data suggest that AR blockade alters androgen metabolism in the castration-resistant VCaP xenograft tumors by a yet unidentified mechanism, and further studies are needed to elucidate if these findings translate to clinical CRPC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

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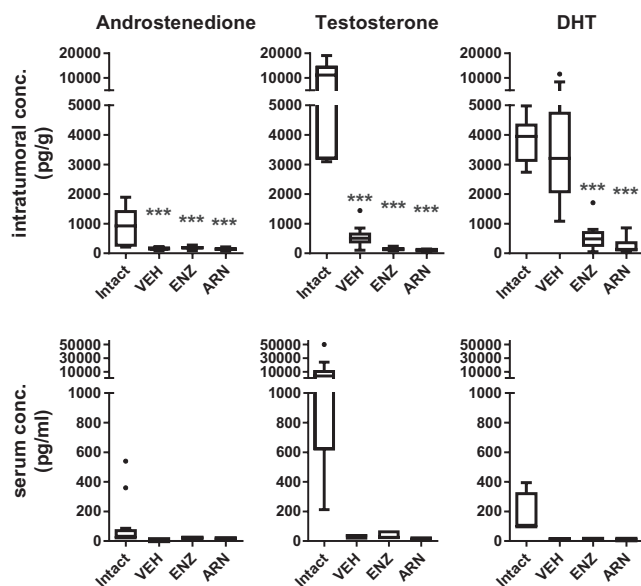


Figure 3

Intratumoral and serum androgen concentrations of VCaP tumors measured by GC-MS (modified from Knuuttila *et al.* 2014 and 2018a). ARN, castration-resistant tumors treated for 4 weeks with ARN-509 (20 mg/kg/day); ENZ, castration-resistant tumors treated for 4 weeks with enzalutamide (20 mg/kg/day); Intact, tumors grown in intact mice; VEH, castration-resistant tumors.

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Received in final form 28 January 2019

Accepted 4 February 2019